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Rosmarinic acid accumulation in *Melissa officinalis* shoot cultures is mediated by ABA

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Abstract

Plant responses to elicitors are the result of a series of highly modulated consecutive changes in hormones or reactive oxygen species (ROS). Absciscic acid (ABA) is a stress hormone that coordinates the complex networks of stress responses and its content is rapidly changed in response to stresses. This study evaluated the effects of application of ABA (0, 5, 25, 50, and 100 μ M) to shoot cultures of lemon balm (*Melissa officinalis* L.) in Murashige and Skoog (MS) liquid medium on growth, H_2O_2 production, rosmarinic acid (RA) content, total phenolic compound accumulation, phenylalanine-ammonia lyase (*PAL*) gene expression, and *PAL* activity. Our results showed that all the applied concentrations of ABA decreased the growth rate of shoots. Moreover, the expression of *PAL*, tyrosine aminotransferase (*TAT*), and rosmarinic acid synthase (*RAS*) genes, *PAL* activity, and the accumulation of total phenolic compound as well as RA were increased in the ABA-treated shoots. The highest content of RA was detected in the shoots treated with 100 μ M ABA. The results suggested that both the *PAL*- and *TAT*-derived pathways were induced by ABA to increase RA accumulation in the shoot cultures of lemon balm. The results revealed that application of ABA led to up-regulation of respiratory burst oxidase homolog (*RBOH*) expression, which was correlated with the production of H_2O_2 in the shoots cultures. In addition, the *cis* epoxy carotenoid dioxygenase (*NCED*) gene, which encodes key enzyme involved in ABA biosynthesis was up-regulated. These results demonstrated that ABA treatment enhanced endogenous ABA content and rosmarinic acid synthesis in the shoot cultures of lemon balm.

Additional key words: 9-*cis* epoxy carotenoid dioxygenase, lemon balm, phenylalanine-ammonia lyase, rosmarinic acid synthase, tyrosine aminotransferase.

Introduction

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. As a main active phenolic compound, it is commonly found in species of the subfamily *Nepetoideae* of the *Lamiaceae*, including perilla, rosemary, sage, mint, basil, and lemon balm (*Melissa officinalis* L.). The RA shows antiviral, antibacterial, anti-inflammatory, hepatoprotective, antiangiogenic, antidepressant, antineurodegenerative, antiallergic, and antioxidant activities (Petersen and Simmonds 2003). The biosynthesis of rosmarinic acid has been vastly investigated for two reasons: 1) rosmarinic acid has been shown to be a useful compound in the field of medicine, and as a food additive, 2) accumulated RA contributes to the defense against microbes. Moreover, the challenge to elucidate the compelling biosynthesis of RA

which consists of two parallel biosynthetic pathways that should be regulated in a coordinated way is one of the other reasons for extensive studying of rosmarinic acid (Matsuno *et al.* 2002). Additionally, rosmarinic acid can be accumulated in plants as a defensive compound and protection against herbivores.

The initial report on the biosynthetic route of rosmarinic acid has demonstrated the involvement of two aromatic amino acids, L-tyrosine (tyrosine-derived pathway) and L-phenylalanine (phenylpropanoid pathway) (Ellis and Towers 1970). A coumaroyl-CoA is derived from phenylalanine in three well characterized enzymatic steps catalyzed by phenylalanine-ammonia-lyase (*PAL*), cinnamic acid 4-hydroxylase (*C4H*), and 4-coumaric acid-CoA ligase (*4CL*). The second pathway

Submitted 7 August 2018, last revision 11 October 2018, accepted 5 November 2018.

Abbreviations: ABA - absciscic acid; MS - Murashige and Skoog; *NCED* - 9-*cis* epoxy carotenoid dioxygenase; *PAL* - phenylalanine-ammonia lyase; RA - rosmarinic acid; *RAS* - rosmarinic acid synthase; *RBOH* - respiratory burst oxidase homolog; ROS - reactive oxygen species; *TAT* - tyrosine aminotransferase.

Acknowledgment: The authors are grateful for the grant from Shahrekord University No. 94GDR1M1032.

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originates from tyrosine which results in the formation of 4-hydroxyphenyllactate (4HPL). Tyrosine aminotransferase (TAT) is the first enzyme in the tyrosine-derived branch of RA biosynthesis. The two ingredients of the parallel pathways, *i.e.*, caffeoyl-CoA and 3,4-dihydroxyphenyllactic acid (DHPL) are connected by rosmarinic acid synthase (RAS) catalyzing a transesterification reaction (Petersen 1991). Kim *et al.* (2013) have pointed out that addition of methyl jasmonate (MJ) into cell suspension culture of *Agastache rugosa* rapidly induced PAL but not TAT activity during RA accumulation. Similar results have been reported by Mizukami *et al.* (1992) who elicited the *Lithospermum erythrorhizon* cell suspension cultures by yeast extract. Conversely, some other reports have demonstrated that elicitors induced biosynthesis of RA and phenolic compounds in *Salvia miltiorrhiza* hairy roots, which was correlated with tyrosine-derived pathway (Yan *et al.* 2006).

The production of phenolic secondary metabolites can be maximized by elicitors, such as MJ, salicylic acid (SA), ABA, polyamines, metal ions, H₂O₂, UV-B, and yeast extracts (Ma *et al.* 2013). Plants respond to elicitor due to a series of highly modulated consecutive changes in their

hormones or ROS (reactive oxygen species) (Jabs 1999, Ton *et al.* 2009). The ABA has been recognized as a stress hormone coordinating the complex networks of stress responses (Zhang *et al.* 2006). In some plant species, changes in the amount of endogenous ABA may play an important role in the induction of biosynthesis of RA and related phenolic compounds (Vagner *et al.* 1998, Gagne *et al.* 2011, Hao *et al.* 2012, Villalobos-Gonzalez *et al.* 2016). Further evidence supports the idea that ABA content is highly modulated by regulation of the different steps of its biosynthesis (Thompson *et al.* 2007, Rodriguez-Gacio *et al.* 2009). Several recent studies have investigated the capability of ABA to induce the accumulation of phenolic compound in cell and tissue cultures as well as in field experiments (Gagne *et al.* 2011, Ibrahim and Jaafar 2013, Liang *et al.* 2013, Villalobos-Gonzalez *et al.* 2016, Murcia *et al.* 2017).

The aim of this study was to investigate the effect of ABA on RA accumulation in the shoot cultures of *Melisa officinalis* in order to reveal the contribution of two metabolic pathways to RA biosynthesis. Further, the correlations between enzyme activities and the expression of corresponding genes were determined.

Materials and methods

Plants, culture conditions, and ABA treatment: Seeds of *Melissa officinalis* L. were purchased from Pakanbazar seed company (Isfahan Province, Isfahan, Iran). Seeds were surface sterilized with 70 % (v/v) ethanol for 20 s and 20 % (m/v) sodium hypochlorite solution for 8 min. Then, they were rinsed five times in sterile distilled water. For germination, seeds were placed in autoclaved hormone free of 1/2 Murashige and Skoog (1962; MS) medium supplemented with 13 g dm⁻³ sucrose and 0.7 % (m/v) agar. After germination, seedlings were maintained in a growth chamber (Binder, Tuttlingen, Germany) at a temperature of 25 ± 1 °C, a 16-h photoperiod, and an irradiance of 650 µmol m⁻² s⁻¹. After one month, plantlets were subcultured using the same medium and conditions. The shoots (5 cm length with 3 nodes) were excised from 60-d-old sterile seedlings and were cultured on hormone free liquid 1/2 MS medium supplemented with various concentrations (0, 5, 25, 50 and 100 µM) of ABA. Stock solution of (±)-abscisic acid (*Sigma*, Steinheim, Germany) was prepared by dissolving certain amounts of ABA in methanol. The same volume of methanol was added to the control shoot cultures. During the experiment, shoot cultures were maintained on an orbital shaker (110 rpm) (Lab Tech, Seoul, South Korea) at conditions mentioned above. The shoots were harvested 7 and 14 d after ABA treatment. Gene expression analysis, H₂O₂, and ABA content were performed on day 7. Shoot fresh mass, total phenolic compound, rosmarinic acid content, and PAL activity were recorded two weeks after elicitation.

H₂O₂ assay: The content of H₂O₂ was measured according to Alexieva *et al.* (2001). Fresh shoot tissue (0.1 g) was macerated in 1.5 cm³ of 0.1 % (m/v) trichloroacetic acid (TCA). After centrifugation (12 000 g, 4 °C, 15 min), 0.5 cm³ of the supernatant was then mixed with 0.5 cm³ of 10 mM potassium phosphate buffer (pH 7) and 1 cm³ of 1 M KI. The absorbance of mixture was measured at 390 nm using a UV/Vis spectrophotometer (Ultrospec™ 3100 pro, GE Healthcare Life Sciences, Pittsburgh, USA). Results were calculated with help of a H₂O₂ standard curve.

Total phenolic and rosmarinic acid content: Total phenolic content was quantified using Folin-Ciocalteu assay (Singleton and Rossi 1965). Plant tissue (0.1 g) was homogenized in 3 cm³ of extraction buffer containing 80 % (v/v) methanol in a pre-chilled mortar. The homogenate was centrifuged at 15 000 g for 15 min. In reaction test tube, 0.02 cm³ of the sample extract was mixed with 0.12 cm³ 20 % (m/v) Na₂CO₃ and 0.15 cm³ of Folin-Ciocalteu reagent. After 30 min at dark and room temperature, absorbance was measured measure at 765 nm. Total phenolic content was expressed as mg of gallic acid per g of fresh mass.

Rosmarinic acid extraction and analysis followed the methods described by Öztürk *et al.* (2010). Methanol extract of shoots (0.2 cm³) was added to 0.2 cm³ of 0.5 M ZrOCl₂ · 8 H₂O and 4.6 cm³ of ethanol. After 5 min, the absorbance of reaction mixture was measured at 362 nm.

Rosmarinic acid (*Sigma*) at the range of 0 - 0.004 mM was used as a standard.

PAL activity analysis: PAL activity was assayed according to Abell and Shen (1987). The reaction mixture contained 2.5 cm³ of 0.1 M Tris-HCl (pH 8.5) with 12 mM phenylalanine and 0.5 cm³ of enzyme extract. The reaction mixture without phenylalanine was added as controls. At the onset of reaction and an hour later, increase in absorbance due to PAL activity was recorded at 290 nm. One unit was defined as the amount that caused an increase of 0.01 in the absorbance per h.

HPLC analysis of ABA content: Quantification of ABA was performed using an HPLC system (*Waters*, Massachusetts, USA) based on the methods of Kelen *et al.* (2004) with slight modifications. For extraction of ABA, 1 g of fresh shoots was homogenized with 10 cm³ of 80 % (v/v) methanol containing butylated hydroxytoluen and ascorbic acid and stirred overnight at 4 °C. The extract was filtered through a *Whatman* filter paper and the methanol was evaporated under vacuum at -35 °C. The residue was dissolved in 0.5 M potassium phosphate buffer, and adjusted to pH 8.5 with 0.2 M KOH. The ethyl acetate was added in volume equal to volume of mixture. The solution was vortexed and the ethyl acetate phase was discarded and the remaining ethyl acetate was freeze dried by evaporating at -35 °C. The pH of the aqueous phase was adjusted to 2.5 with 0.2 M HCl. The residue was dissolved in ethyl acetate, and the extract was evaporated under vacuum to remove ethyl acetate. The dry residue was dissolved in 0.5 cm³ of methanol and filtered through a 0.22 µm membrane and stored at -80 °C until analysis. The samples (0.02 cm³) was injected into a reverse-phase column (C18, 250 × 4.6 mm). The mobile phase consisted of acetic acid (0.2 %) and methanol (100 %) (50:50, v/v), and the flow rate was 0.7 cm³ min⁻¹. Apparatus wavelength was adjusted to 265 nm. Identification of unknown peaks was based on retention time and UV absorption spectral comparison with known standards of (±)-ABA.

RNA extraction and cDNA synthesis: RNAs were extracted by *DENAzist* kit (*DENAzist Asia Co.*, Mashhad, Iran) from fresh leaves according to manufacturer's instructions. The recombinant DNase I treatment was used to remove contaminating DNA from RNA preparations and RNA (500 ng) was reverse transcribed to first strand cDNA using *PrimeScript RT Enzyme Mix I* (*TaKaRa Bio*, Otsu, Japan), which were later used as templates for real-time PCR.

The sequences of the *RAS* and *PAL* genes were

obtained from the *NCBI* database (GenBank accession Nos. FR670523.1 and FN665700.1, respectively). The primers of *NCED* (9-*cis* epoxy carotenoid dioxygenase) corresponding to conserved regions of *NCED* were determined using a nucleotide alignment that included *NCEDs* from a range of plant species: *Sesamum indicum* (XM-011084544.2), *S. indicum* (XM-011099822.2), *Scutellaria baicalensis* (KC760149), *Fragaria × ananassa* (JX013945.1), *Fragaria vesca* (XM-004300619.2), *Erythranthe guttatus* (XM-012981017.1), and *Daucus carota* (NM-001329172.1). The primers for *Actin* and *TAT* genes were taken from Doring *et al.* (2014). RNA-seq data (GSE100970) was used for design primers of *RBOH* gene (NADPH oxidase B).

Real-time quantitative PCR analysis: Relative gene expression was determined by real-time qPCR using a *Rotor-Gene Q* instrument (*Qiagen*, Hilden, Germany). The PCR mixture (0.01 cm³) contained 10 µM of each primer, 50 ng of the cDNA template and 0.005 cm³ of *SYBR Premix Ex Taq* (*TaKaRa Bio*). The thermal cycle conditions were as follows: pre-incubation at 95 °C for 10 min, 40 cycles of 95 °C for 5 s, 58 - 62 °C for 10 s, and 72 °C for 20 s. The purity of the amplified products was confirmed by melting curve analysis and agarose gel electrophoresis. All reactions were performed twice.

Data analysis: The data were analyzed by *ANOVA* in the computer software *SAS v. 8*. Mean comparisons were performed with the least significant difference (LSD) test.

Fig. 1. Effect of different concentrations of abscisic acid (ABA) on fresh mass of *Melissa officinalis* shoots (SFM) two weeks after application. Means $\pm$ SDs, $n = 3$. Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test.

Results

The shoots fresh mass (SFM) was significantly affected by ABA at all applied concentrations with the maximum

reduction in the shoots treated with 100 µM of ABA (Fig. 1). Evaluation of H₂O₂ production (Fig. 2A) indicated

a significant enhancement of H_2O_2 content in all ABA-treated shoots with maximum at the highest ABA concentration. Also, the activity of PAL was increased with increasing ABA concentration (Fig. 2B). The content of phenolic compounds was 1.09-, 3.24-, 1.5-, and 1.7-fold higher in the shoots exposed to 5, 25, 50, and 100 μM of ABA, respectively, compared to the control (Fig. 3A). Similarly, rosmarinic acid content was a significantly

enhanced in all the ABA-treated shoots compared to the control. The highest content of rosmarinic acid was detected in the shoots treated with 100 μM ABA two weeks after elicitation, which was 14 times higher than that of the control (Fig. 3B). One week after elicitation with 50 μM of ABA, a 55-fold increase was observed in the endogenous ABA content, which was the maximum amount of ABA detected in the elicited shoots (Fig. 4).

Fig. 2. Effect of different concentrations of ABA on H_2O_2 production (A) and PAL activity (B) in *Melissa officinalis* shoots one weeks after application. Means $\pm$ SDs, $n = 3$. Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test.

Fig. 3. Effect of different concentrations of ABA on content of phenolic compounds (A) and rosmarinic acid (B) in *Melissa officinalis* shoots one week after elicitation. Means $\pm$ SDs, $n = 3$. Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test.

The effects of various concentrations of ABA on expressions of *PAL*, *TAT*, *RAS*, *RBOH*, and *NCED* genes were investigated using real-time PCR analysis (Fig. 5). Evaluation of gene expression profile indicated that *PAL* was significantly up-regulated in all the ABA-treated shoots. The expressions of *TAT* gene were significantly up-regulated at the ABA concentrations of 25, 50, and

100 μM compared to the control. Moreover, gene expressions of both *RAS* and *RBOH* genes were up-regulated at all concentrations of ABA compared to the control, but the highest expression of *RBOH* gene was detected in the shoots treated with 25 μM ABA. The expression of *NCED* gene was significantly up-regulated in the shoots treated with 25 and 50 μM of ABA.

Discussion

The present study investigated whether ABA-application alters expressions of the genes involved in the biosynthesis of RA and its accumulation in the shoot cultures of lemon balm. ABA is often described as a growth inhibitor since the reduced growth under stress condition is correlated with the increased ABA content; moreover, applied ABA prevents germination and seedling growth (Hedden and Thomas 2006). In this study, substantial differences in growth rate were observed between the ABA-treated and control shoots.

Fig. 4. Effect of different concentrations of ABA on endogenous ABA in *Melissa officinalis* shoots one week after application. Means $\pm$ SDs, $n = 3$. Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test.

Generally, treatment by various types of elicitors induces a rapid increase in the endogenous ABA content (Gagne *et al.* 2011, Hao *et al.* 2012, Villalobos-Gonzalez *et al.* 2016, Kuyyogsuy *et al.* 2018). Therefore, both the content of endogenous ABA and the expression of pivotal gene associated with the ABA biosynthetic pathway (*NCED*) increased in the shoot cultures of lemon balm treated with ABA. The ABA content in shoots was increased within 7 d after ABA application and its maximum was detected in the shoots treated with 50 μ M ABA. Increase in the expression of *NCED* leads to an increase in the ABA content (Castellarin *et al.* 2016) also in response to environmental stresses (Agusti *et al.* 2007, Kuyyogsuy *et al.* 2018). In our study, the expression of *NCED* was up-regulated in the plants treated with 50 μ M ABA compared to the control. Results showed that although expression of *NCED* was not increased in the shoots treated with 5 μ M ABA, the endogenous ABA was increased in the shoots, suggesting that the increase could be due to the decreased catabolism of this hormone. Our finding revealed that the ABA application could induce an increase in the endogenous ABA together with *via* up-

regulation of the *NCED* gene expression. Thus, it could be suggested that the exogenous ABA stimulated ABA biosynthesis through increasing expression of *NCED*.

The applied ABA can also trigger the generation of H_2O_2 in the plant cells or tissues (Hu *et al.* 2010, Ye *et al.* 2011). ROS, especially H_2O_2 and $O_2^{\cdot-}$, are involved in the ABA signaling pathway, thus, ABA increases ROS accumulation which leads to changes in gene expression and in metabolism. After foliar applications of ABA antioxidative properties of cells might be up-regulated by the increase in the content of phenolics and flavonoids (Ibrahim and Jaafar 2013). Accumulation of ROS in plants is generally attributed to several possible sources, including plasma membrane-localized NAD(P)H oxidase, oxalate oxidase, apoplastic peroxidase, and amino oxidase (Bolwell 1999). Previous studies have found that the H_2O_2 generated by NADPH oxidase encoded by *RBOH* plays an important role in plant responses to biotic and abiotic stresses. The results of the present study indicated that the expression of *RBOH*, which is a key player in controlling H_2O_2 accumulation, was significantly up-regulated by ABA application.

Phenylalanine ammonia lyase (PAL) is known as a key enzyme in RA biosynthetic pathway (Dong *et al.* 2010). Several studies have revealed that exogenous ABA could induce the activation of PAL (Hao *et al.* 2012). The results of the present study revealed that exogenous ABA induced the activation of PAL and synthesis of RA in the shoots of lemon balm. Here, we reported that ABA induced the accumulation of RA and phenolic compounds. Our findings are consistent with those of the previous studies on other plant species which reported that the content of RA and phenolic compounds was increased by application of biotic and abiotic elicitor (Yan *et al.* 2006, Bauer *et al.* 2009, Kim *et al.* 2013, Ejtahed *et al.* 2015). In this study, ABA was used as an elicitor to examine the relationship between the expression of rate-limiting enzymes in the two RA biosynthesis pathways and accumulation of RA in the shoot cultures. The expressions of *PAL*, *TAT*, and *RAS* genes were significantly up-regulated as the result of ABA treatment. Expressions of these genes are correlated with the RA content in several plants (Weitzel and Petersen 2010, Doring *et al.* 2014). These results suggested that both the PAL- and TAT-derived pathways were induced by ABA to increase RA accumulation in the shoot cultures of lemon balm.

In summary, ABA application could effectively induce RA accumulation by the induction of the PAL activity, as well as by the up-regulation of *PAL*, *TAT*, and *RAS* genes. In addition, the up-regulated expression of *NCED* gene by ABA treatment is correlated with the enhancement of ABA content. In this study, ABA not only mediates the accumulation of endogenous ABA, but also controls the production of H_2O_2 by changing the expression pattern of *RBOH* gene.

Fig. 5. Relative transcriptions of *PAL* (A), *TAT* (B), *RAS* (C), *NCED* (D), and *RBOH* (E) genes in *Melissa officinalis* shoots treated with different concentrations of ABA in relation to the housekeeping gene *actin*. Means $\pm$ SDs, $n = 3$. Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test.

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